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STUDIES ON THE ANTIGENICITY OF AN ACID-PRECIPITATING SUBSTANCE PRODUCED BY BACILLUS ANTHRACIS IN THE CULTURE FLUID

I. ANTIGENICITY OF THE CULTURE FLUID AND ITS FRACTIONS

[Following is a translation of an article by Shizuo Takagi and Takeshi Hironao, Laboratory of Microbiology, Department of Veterinary Science, College of Agriculture, Osaka Prefecture University, Japanese Journal of Bacteriology, Vol. 17, 1962, pages 334-340.]

Preface

Although many authors [1-23] have reported on the antigen of B. anthracis relating to precipitation reaction, primarily on polysaccharid, and sclera d-glutamic acid polypeptides, no report was found on the antigenicity of protein (including polypeptide), except the report by Ivanovic), who obtained an albumose like substance in the process of separation of bacteria-body polysaccharides. He only mentioned that it had no relation to precipitinogenicity. Recently, it was reported [24-33] that proteinic prophylactic antigen was produced in the fluid obtained from the oedematous tissue infected by B. anthracis. However, some people reported that they could not prove the existence of this antigen [26-27]. Others reported that they found it. Thus, we feel that this point is not entirely clarified as yet. Since the study of such protein antigenicity seems significant to clarify the antigen of B. anthracis and its relation to the bacteria with similarity, the present study was performed as follows. Since the extract of heat-dried B. anthracis contained highly common antigenic substance, since the substance obtained from the washed fluid of bacteria body was rather specific, [34) 40) 41)] and since B. anthracis produced prophylactic antigen or poisonous albumose like substance, [32)] culture fluid was chosen for the present study. The culture fluid was divided into acid precipitated protein fraction and supernatant fraction. The antigenicity of fractions was compared to that of the whole culture fluid. A closely related bacteria having a high common antigenicity to B. anthracis in the part of polysaccharide was used as a control.

Experimental Materials and Methods

Bacteria strain used in the experiment: B. anthracis pasteur 1 strain (shared from the Laboratory of Animal Hygiene, Department of Agriculture and Forestry, arbitrarily called Va-1 strain), Sakai strain

(separated from the spleen of a cow which died of anthrax), and Bacillus megaterium strain of Tokyo University (shared by Dr. Yuichi Ochi, Laboratory of Animal Bacteriology, Division of Veterinary, Department of Agriculture, Tokyo University.) This is arbitrarily called the TU strain. This is thought to be closely related to Bacillus cereus of Bergey's classification in its biological characteristics, and it has a highly common antigenic reactivity to B. anthracis.)

Culture medium and culture method: Acid precipitated part in the culture medium was thought important in this experiment. For the analysis of antigen, especially of its chemical structure, it was thought advantageous to eliminate the acid precipitated part having no relation to antigenic substance of the culture medium. Therefore, a thick bouillon containing 1% pepton and meat extract, and 5% NaCl was prepared, adjusting pH at 7.2 with conc. NaOH and then, boiled. Sediment was removed and conc. NaOH was added again to adjust pH at 7.2. Then aqua dest. was added to dilute it 10 times and pH was checked. Each 100 ml of this solution was transferred into Roux's flask (the thickness of the fluid was 5 to 10 mm [milimeter] at the time of culture) and sterilized under high pressure. Agar cultured fresh bacteria was transplanted 1/10 loop each in amount to the above and cultured still for 2 days at 37°C.

Fractionation of culture fluid: After the culture, it was centrifuged (at 4000 rpm) 20 to 30 min. several times, and then at 12,000 rpm for 30 min. several times, to separate bacteria-body and then an aliquot of the supernatant was taken, adjusted at pH 7.2. To the above, [marsonine or marzonin?]* was added ($1:10^4$) to make a culture fluid. To the rest of the supernatant, N-HCl was added to bring pH to 3.8. Then precipitate was separated, washed with (ph. 3.8) citrate buffer, and dissolved in the physiologic saline (pH. 7.2, [marsonine or marzonin?]* added) and brought to the previous volume. The insoluble part was separated by centrifugation and was named acid precipitated fraction. The supernatant from which the above fraction was obtained was treated with N-NaOH to bring pH to 9.2, then [marsonine or marzonin?]* was added; this was named supernatant fraction. To examine antigenicity, each fraction was diluted or concentrated at our convenience. Anti-sera used. One half percent formalin treated dead bacteria of B. anthracis Sakai strain and B. megaterium TU strain was injected to the rabbit by the Takagi method.[3,4]

Table 1. Precipitation reaction of culture fluid (V-0, S-0, M-0) to anti-anthrax and anti-megaterium serum

anti-sera (1:2) and rabbit No.	B. anthracis Va-1 strain V-0		B. anthracis Sakai strain S-0		B. megaterium TU strain M-0	
anti-anthrax						
No. 229	++	32	++	32	++	16
230	++	32	++	32	++	16
232	++	32	++	32	++	32

* Marzonin in Japanese pronunciation and text.

anti-megaterium:						
No. 233	++	16	++	16	++	32
240	++	16	++	16	++	32
262	++	32	++	32	++	32

++, +++; strength of reaction of ½ diluted antigen. number; precipitin titer at various dilution of antigen.

Table 2. Precipitation reaction of acid precipitated fraction (V-A, S-A, M-A) to anti-anthrax and anti-megaterium serum

anti-sera (1:1) and rabbit No.	B. anthracis Va-1 strain V-A	B. anthracis Sakai strain S-A	B. megaterium TU strain M-R
anti-anthrax:			
No. 229	+ 1	+ 1	-
230	+ 1	+ 1	-
232	+ 1	+ 1	-
anti-megaterium:			
No. 232	-	-	-
240	-	-	-
262	-	-	-

-, +; strength of reaction of 1:1 diluted antigen. number: precipitin titer at various dilution of antigen.

Table 3. Precipitation reaction of supernatant fraction (V-R, S-R, M-R,) to anti-anthrax and anti-megaterium serum

anti-sera (1:2) and rabbit No.	B. anthracis Va-1 strain V-R	B. anthracis Sakai strain S-R	B. megaterium TU strain M-R
anti-anthrax:			
No. 229	++ 16	++ 32	+ 16
230	++ 16	++ 32	++ 16
232	++ 32	++ 32	++ 16
anti-megaterium:			
No. 233	++ 16	++ 16	++ 32
240	++ 16	++ 16	++ 32
262	++ 16	++ 32	++ 32

+, ++; strength of reaction of antigen at 1:2 dilution. number: precipitin titer at various dilution of antigen.

Table 4. Absorption test of the culture fluids of B. anthracis, B. megaterium and their fractions

absorption: sera (anti serum 1: antigen 4)*		B. anthracis Sakai strain			B. megaterium TU strain		
		S-O	S-A	S-R	M-O	M-A	M-R
anti-anthrax:							
(No. 232)	M-O	+	+	-	-	-	-
	M-A	+	+	+	+	-	+
	M-R	+	+	-	-	-	-
control physiol. saline		++	+	++	++	-	++
anti-megaterium:							
(No. 262)	S-O	-	-	-	++	-	++
	S-A	+	-	+	++	-	++
	S-R	-	-	-	++	-	++
control physiol. saline		++	-	+	++	-	++

* antigen was condensed two times

Table 5. Absorption test of the culture fluid of B. anthracis Sakai strain and its fraction with anti-anthrax and anti-megaterium serum

absorption antigen (antigen 1 : anti-serum 5)*	anti-anthrax sera			anti-megaterium sera		
	No.229	No.230	No.232	No.233	No.240	No.262
S-O:						
anti-anthrax	-	-	-	-	-	-
anti-megaterium	+	+	+	-	-	-
normal control	++	++	++	++	++	++
S-A:**						
anti-anthrax	-	-	-	-	-	-
anti-megaterium	+	+	+	-	-	-
normal control	+	+	+	-	-	-
S-R:						
anti-anthrax	-	-	-	-	-	-
anti-megaterium	-	-	-	-	-	-
normal control	++	++	++	+	++	++

* anti-anthrax serum No. 232, and anti-megaterium serum No. 262.
** original serum was condensed 7 fold.

Precipitation reaction and absorption test: Following the previously reported layering method, intra-agar reaction was performed by the Willson and Pringle method. [42] The extract of agar cultured and heat-dried bacteria was used as the control of each bacteria, as described in the previous paper. [40] The absorption test was carried out taking Kabat and Mayer's [39] description into account.

Other tests: Qualitative tests applying routine Biuret, Ninhydrin and Molisch's reaction were performed at various concentrations of each part of the culture fluid.

Results

Antigenicity of culture fluid is shown in Table 1. It can be assumed that there were common antigenic substances in the culture fluids of these bacteria, since B. anthracis and B. megaterium showed considerable reactivity to their anti-serum each other as well as to their antiserum. Acid precipitated fraction is shown in Table 2. Acid precipitated fraction of B. anthracis reacted to anti-anthrax serum, but did not react to anti-megaterium serum. The fraction of B. megaterium did not react to any of anti-serum. Therefore, the acid precipitated fraction of B. anthracis has antigenicity as precipitinogen. Moreover, it is thought to be different from B. megaterium's.

Supernatant fraction as shown in Table 3, showed almost the same results as culture fluids, except for a very small difference. It seemed possible that the common antigenic substance was included in the fraction of both B. anthracis and B. megaterium. Then an absorption test was performed to observe the precipitinogenicity of the culture fluid of bacteria and their two fractions.

As shown in Table 4, each antigen part was absorbed from each antiserum. The antigen to supernatant was mostly removed in the case of anti-anthrax serum, but the antigen to culture fluid and acid precipitated fraction remained after the absorption with each part of B. megaterium. However, there observed no antigenicity of the acid precipitated fraction of B. megaterium in the experiments. Therefore, it showed no relation with other parts in the absorption test. Although in the case of anti-megaterium serum, the antibody for B. anthracis was removed when it was absorbed with each part of B. anthracis and the antibody to the culture fluid and supernatant fraction seemed to remain. There will be different kinds of antigenic substances in the culture fluid of B. megaterium from those of B. anthracis, therefore they may not be precipitated with acid. Since anti-megaterium serum did not react to the acid precipitated fraction of B. anthracis, no relation with other parts was seen in the absorption test. According to the results, it is naturally understood that a considerable amount of the common antigen parts are included in the culture fluid of B. anthracis and B. megaterium and these migrate to the supernatant fraction which does not precipitate with the acid. Although it can be known that the acid precipitated fraction of B. anthracis may be different from that of B. megaterium, another absorption test was carried out on this point. The absorption test with

anti-anthrax and anti-megaterium serum using each part of B. anthracis Sakai strain is shown in Table 5. In the culture fluid, there contained the antigen part which could not be removed by the antibody in anti-megaterium serum. Such results were obtained also in the acid precipitated fraction. Of course the supernatant fraction was entirely absorbed with anti-anthrax serum and with the antibody in the anti-megaterium serum. Therefore, this fraction is supposed to be composed mostly of the common antigenic part.

The above results of B. anthracis Sakai strain was studied with intra-agar precipitation reaction. The culture fluid and the supernatant both reacted to anti-anthrax serum (Fig. 1, I) and anti-megaterium serum (Fig. k, II). Moreover, it formed a precipitation belt showing common reaction with the same kind of control bacteria and with bacteria of similarity. The culture fluid and the acid precipitated fraction formed another precipitation belt only with anti-anthrax serum (Fig. 1I, and fig. 1III). Although the figure is not shown here the belt of acid precipitated fraction continued to the fine precipitation belt observed in the previous culture fluid. About the same results were obtained in Va-1 strain of B. anthracis. A simple chemical qualitative test was done for each part used in the experiment. Biuret, Ninhydrin, and Molisch reactions were positive for the culture fluid of each bacteria. Biuret and Ninhydrin were positive and the Molisch reaction was almost negative to the acid precipitated fraction. To the supernatant fraction, Biuret was negative and Ninhydrin and Molisch reactions were positive. From the above results, it is supposed that the part consisted mainly of protein is contained in the acid precipitated fraction, and polysaccharide is contained in the supernatant fraction.

Summary and Comment

B. Anthracis Va-1 strain, Sakai strain and B. megaterium TU, having a very high common antigenicity and similarity to the first two strains were cultured in the bouillon from which an acid precipitated ingredient was removed. The three fractions, their culture fluid, acid precipitated and supernatant fraction were studied on their precipitinogenicity and mutual relationship by layering method, intra-agar precipitation reaction, and absorption test. The culture fluid of B. anthracis and B. megaterium contained the common antigenic parts reaction to its own and to other anti-serum as well. This part migrated into the supernatant fraction, which did not precipitate with acid. This fraction showed Biuret negative and Molisch positive reactions qualitatively. Therefore, this may contain a common antigenic polysaccharide as reported in the previous paper. 34-36) The acid precipitated fraction of B. anthracis showed precipitation reaction with anti-anthrax serum. Therefore, it was known that this fraction had precipitinogenic property. This fraction showed positive protein reaction and it had entirely different antigenicity from supernatant fraction. This fraction never reacted with anti-megaterium serum in the experiment, as if it had no antigenicity. However, this may due

to the low concentration of antigen used in the experiment. Therefore, further study will be done on this point at the next opportunity.

Summary

1). Antigenic substances showing common reaction to B. megaterium TU, and other substances showing different antigenicity were contained in the culture fluid of B. anthracis.

2). Acid precipitated fraction contained protein and it showed reactivity as an precipitinogen. This is a different antigenicity from B. megaterium.

3). The fraction which did not precipitate with acid consisted mainly of polysaccharide. It demonstrated the existence of antigenic substance having a common reaction to B. megaterium.

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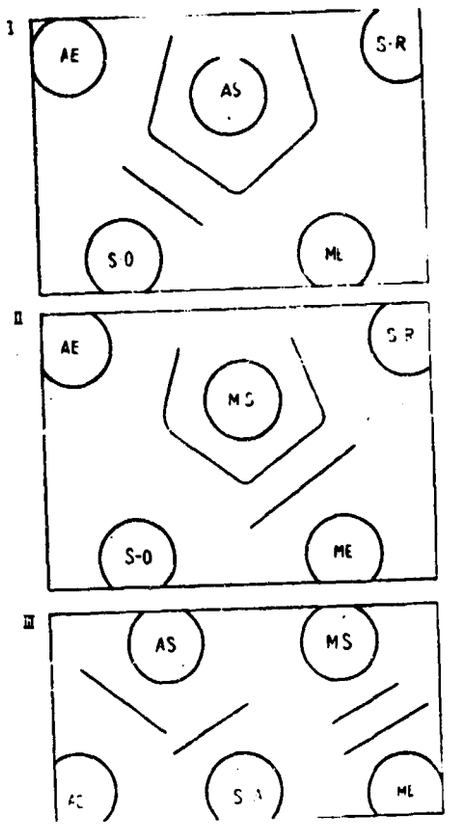

Fig 1. Intra-agar precipitation reaction of the culture fluid and fractions of B. anthracis Sakai strain.

S-O-- culture fluid
S-R-- supernatant fraction
AS--- anti-anthrax serum
S-A-- acid precipitated fraction concentrated three times
AE--- extract of heat-dried B. anthracis (control, $1:10^2$)
MS--- anti-megaterium serum